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Gene-specific disruption of endocannabinoid receptor 1 (*cnr1a*) by ethanol probably leads to the development of fetal alcohol spectrum disorder (FASD) phenotypes in Japanese rice fish (*Oryzias latipes*) embryogenesis

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ABSTRACT

The present study was designed to investigate the probable roles played by cannabinoid (CB) receptors in fetal alcohol spectrum disorder (FASD) induction in Japanese rice fish (*Oryzias latipes*). Searching of public databases (GenBank, Ensembl) indicated that the Japanese rice fish genome includes three human ortholog CB receptor genes (*cnr1a*, *cnr1b* and *cnr2*). Quantitative real-time PCR (qPCR) and whole mount in situ hybridization (WMISH) techniques were used to analyze the expression of these *cnr* genes during Japanese rice fish embryogenesis and also in response to developmental ethanol exposure. qPCR analyses showed that the expression of all three CB receptor genes were developmentally regulated and only *cnr2* showed maternal expression. The mRNA concentrations of these genes were found to be enhanced after 3 dpf and attained maximal levels either prior to or after hatching. WMISH technique indicated that all three cnr genes were expressed in the head region of hatchlings. During development, ethanol selectively attenuated the expression of *cnr1a* mRNA only. Blocking of *cnr1a* mRNA by CB1 receptor antagonists rimonabant (10–20 μM) or AM251 (0.2–1 μM) 0–2 dpf were unable to induce any FASD-related phenotypic features in embryos or in hatchlings. However, continuous exposure of the embryos (0–6 dpf) to AM251 (1 μM) was able to reduce the hatching efficiency of the embryos. Our data indicated that in Japanese rice fish, ethanol disrupted the expression of only *cnr1a* in a concentration-dependent manner that induced delay in hatching and might be responsible for the development of FASD phenotypes.

1. Introduction

Alcohol is a systemic toxin that exerts complex effects on human physiology and health. Alcohol abuse and dependency, together known as alcohol use disorder (AUD), affects an estimated 8.5% of the US population over the age of 18 (NIAAA, 2009). Alcohol is not only an addictive and neurotoxic, but also a fetal teratogen, and an etiologic agent in hepatic and cardiovascular diseases, inflammation, bone loss, and fracture susceptibility (Miranda et al., 2010). Epidemiological, experimental and clinical investigations have shown a strong relationship between alcohol abuse and cardiomyopathy, hypertension, brain anomalies and other disorders (Zakhari, 1997; Klatsky, 1999). AUD costs the US economy up to $185 billion per year (NIAAA, 2009). Consumption of alcohol during pregnancy can produce a wide range of irreversible cognitive, behavioral, structural and physical anomalies known as fetal alcohol spectrum disorder (FASD) (Finn and Justus, 1997; Goodlett et al., 2005). Fetal alcohol syndrome (FAS) is the most clinically recognizable form of FASD which is characterized by a set of facial dysmorphogenesis, mental dysfunction, growth retardation and cardiovascular and limb defects. FASD is recognized as the principal known cause of mental retardation in the USA (Abel and Sokol, 1992). It is estimated that about 1 to 3% of children are born with FASD, but the incidence of FAS in the USA is 1–2 per 1000 live births (Cook et al., 1990; Sampson et al., 1997). Although a large number of genes and signaling mechanisms have been implicated in alcohol's deleterious effects (Bora and Lange, 1993; Aroor and Shukla, 2004; Nunez and Mayfield, 2012), the molecular mechanisms of FASD are yet unknown. Evidence-based data on animal experiments demonstrated that alcohol (ethanol) interferes with many ontogenic phases of brain development, affecting neuronal migration, neurogenesis, and gliogenesis (Guerri, 2002).

Over the past fifty years, a wealth of data obtained from animal and human studies suggested an interaction between endocannabinoid signaling (ECS) and alcohol dependence (Basavarajappa and Hungund, 2005; Pava and Woodward, 2012). ECS comprises cannabinoid receptors, endogenous ligands and enzymes involved in the biosynthesis and degradation of these ligands and putative membrane transport proteins (Erdozain and Callado, 2011). Two major cannabinoid receptors, CB1 (CNR1) and CB2 (CNR2) belonging to the large family of seven

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transmembrane-spanning G-protein-coupled receptors (GPCRs) have been identified (Matsuda et al., 1990; Howlett et al., 2002). CNR1 is widely distributed in several regions of the brain, with a high density in the cortex, hippocampus, basal ganglia and cerebellum and is known to regulate neurotransmitter release (Wilson and Nicoll, 2002). CNR2 is mainly located in peripheral tissues of the immune system (Munro et al., 1993). Genes encoding orthologs of the mammalian *Cnr1* are found throughout vertebrates including chicken, turtle, frog, and fish (Elphick and Egertova, 2001). Pharmacological actions of cannabinoids and specific binding sites for cannabinoids have been reported in several invertebrate species, but the molecular basis for these effects is not known (Elphick and Egertova, 2001). Biochemical, behavioral, and genetic studies using in vitro, in vivo, and human brain data indicated that both chronic and acute administration of alcohol produced alterations in different elements of ECS (Erdozain and Callado, 2011). The activation of CNR1-mediated signaling is a potential mechanism by which ethanol alters early brain development and is therefore regarded as an important candidate for the pathophysiology of FASD. In C57BL/6J mice, ethanol treatment during postnatal day 7 increased AEA/CNR1 signaling and resulted in neonatal neurodegeneration that contributes to the development of synaptic and memory deficits relevant to FASD (Subbanna et al., 2013). A recent report indicates that ethanol exposure of C57BL/6J mice during neurodevelopment decreased the expression of *Cnr1* mRNA and increased the expression of regulatory microRNA *miR-26b* in adult brain which might be a potential cause of FASD (Stringer et al., 2013). The response of the *Cnr2* gene to ethanol is unclear. Interestingly, vertebrates in lower orders of evolution such as fish have paralogous *cnr* genes, and their response to ethanol is also unknown. The presence of more than one *cnr* gene (paralogs) in fish genome is also of particular interest because it is likely to be a consequence of a whole genome duplication event that is thought to have occurred after the divergence of ray-finned fish and lobe-finned fish (Amores et al., 1998). Therefore, it is important to investigate whether *cnr* has acquired different functions during evolution or is simply co-expressed and co-functional in a common population of cells in fish.

Like mammalian models, fish models, particularly zebrafish and Japanese rice fish, because of their easy availability, low maintenance cost, short life cycle and also their accessibility to the study of gene functions (Furutani-Seiki and Wittbrodt, 2004) are currently emerging as an alternative to study FASD, and have contributed significantly to our understanding of the molecular mechanisms of FASD. We have been using Japanese rice fish as an animal model of FASD and reported several analogous FASD phenotypic features in the cardiovasculature and neurocranium induced by developmental ethanol exposure (reviewed by Haron et al., 2012) which are also observed in humans and other experimental animals. Therefore, the present study is designed to see whether ethanol while inducing FASD phenotypes is able to target any of the *cnr* genes that are expressed in Japanese rice fish embryogenesis. There are three *cnr* gene sequences included in the Japanese rice fish genome database (two paralogous *cnr1*: we named them *cnr1a* and *cnr1b*, and one *cnr2*). Our aim is to investigate the response of *cnr* genes to developmental ethanol exposure and find out whether *cnr* genes play any role in FASD induction. Our data indicate that in Japanese rice fish, ethanol, while inducing FASD phenotypes, specifically disrupts the expression pattern of *cnr1a* gene; the other two CB receptor genes (*cnr1b* and *cnr2*) maintain their normal developmental rhythm in the presence of ethanol. Therefore, if CB receptors play any role in FASD induction, it may be mediated through *cnr1a* rather than *cnr1b* or *cnr2*.

2. Materials and methods

2.1. Experimental procedure

The Institutional Animal Care and Use Committee (IACUC) of the University of Mississippi (UM) approved all the experimental protocols.

Methods of animal maintenance, collection of fertilized eggs, identification of the different developmental stages (Iwamatsu, 2004) and the culture conditions of Japanese rice fish embryos in the laboratory were described previously (Hu et al., 2008). In brief, embryos after collection and screening were maintained in hatching solution (17 mM NaCl, 0.4 mM KCl, 0.6 mM $MgSO_4$, 0.36 mM $CaCl_2$ with required amount of $NaHCO_3$ to maintain the pH 7.4 and 0.0002% methylene blue to reduce fungal infection) under a 16L:8D light cycle in a Precision High Performance Incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 26 ± 1 °C. For normal development, the collected embryos were maintained in clear glass bowls (10 × 4.5 cm) in 150–200 ml hatching solution (50–100 embryos/bowl) with 50% static renewal of the media every day. For studying the developmental expression of *cnr* genes, viable 0 (Iwamatsu stages 9–10), 1 (Iwamatsu stages 17–18), 2 (Iwamatsu stages 23–24), 3 (Iwamatsu stages 27–28), 4 (Iwamatsu stages 29–30), and 6 dpf (Iwamatsu stages 34–38) embryos and hatchlings (within 24 h of hatching) were used for RNA extraction (8 embryos or hatchlings/set). To study the effects of ethanol (100–500 mM) on *cnr* gene expression at the message level, viable 0 dpf embryos (Iwamatsu stages 9–10) were transferred to 2 ml tubes (1 egg/tube) in 1 ml medium (hatching solution) containing 100–500 mM ethanol depending upon the nature of the experiments. The tubes were tightly capped to stop evaporative ethanol loss. Control embryos were maintained in 1 ml hatching solutions (1 egg/tube) without ethanol. The media were changed every day. Some of the control and 300 mM ethanol-treated embryos after 2 days of treatment were utilized for RNA extraction. The remaining embryos (control and embryos treated with 100–500 mM of ethanol) were transferred to a 48 well plate and maintained in clean hatching solution (one embryo/well/ml medium) for another 4 days (6 dpf) with 50% static renewal of the media. In a separate experiment, the embryos (0 dpf, Iwamatsu stages 9–10) were exposed continuously to 300 mM of ethanol from 0 to 6 dpf and maintained in tightly capped 2 ml tubes in 1 ml hatching solution with or without (control) ethanol. The medium with ethanol was changed every day. After treatment, the viable embryos were pulled out (6–8 per sample) and homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) for RNA extraction (Dasmahapatra et al., 2005). To remove genomic DNA, the extracted RNA was treated with nuclease-free RQ1 DNase (Promega, Madison, WI) and the concentration of the purified RNA was determined in a Nano Drop (Thermo Scientific, Wilmington, DE). RNA was reverse transcribed to cDNA by iScript supermix (BioRad Laboratories, Hercules, CA) in a 20 μl final volume following manufacturer's protocol. For qPCR analysis, 1 μl of cDNA in duplicates was used for each gene and the data were expressed as mRNA copy/ng RNA (Wu et al., 2010). To determine the spatial pattern of expression of *cnr* mRNAs in specific regions, whole mount in situ hybridization (WMISH) analysis of hatchlings (~24 h post hatch) was used following Inohaya et al. (1995).

2.2. Rimonabant and AM251 exposure

Fertilized eggs (Iwamatsu stage 10) were exposed to two known CB1 receptor antagonists rimonabant (Sigma-Aldrich, St. Louis, MO) or AM 251 (Tocris, Minneapolis, MN) for evaluation of FASD phenotypic features in cardiovasculature or neurocranium of medaka embryos and hatchlings. Embryos (Iwamatsu stage 10) were exposed to 10–20 μM rimonabant or to 0.2–1 μM AM 251 for 48 h in 1 ml hatching solution in 2 ml tight capped tubes. The media with requisite concentrations of antagonists were changed once at 24 h. Control embryos were exposed to 0.01% DMSO (vehicle). After treatment the viable embryos were transferred to 48 well plates (one embryos/well in 1 ml hatching solution) and maintained in clean hatching solution with 50% static renewal of the media every day. The thrombus formation, heart morphology and the onset of vessel circulation were checked under a phase contrast microscope every day. When the embryos were hatched, with in 24 h of hatching, they were preserved in 4% paraformaldehyde

(PFA) followed by Alcian blue staining (Haron et al., 2013). In another set of experiments, fertilized embryos (Iwamatsu stage 10) were exposed to 1 μM of AM 251 for 6 dpf (0–6 dpf with complete renewal of media everyday) and then transferred to 48-well plates (one embryo/well in 1 ml hatching solution), checked for thrombus formation, heart morphology, and vessel circulation, and allowed to hatch. Once embryos hatched, they were preserved in 4% PFA within 24 h of hatching and used for Alcian blue staining. The stained embryos were examined for craniofacial deformities as described previously (Hu et al., 2009).

2.3. Statistics

All data (except six day continuous ethanol exposure experiments) were analyzed by using one way ANOVA followed by post-hoc Tukey's multiple comparison test. For six day continuous ethanol exposure experiments, the mean data were analyzed by using Student's unpaired "t" test. Data were expressed as mean ± SEM of 4–6 independent experiments and $p < 0.05$ was considered significant.

3. Results

3.1. Expression of cnr mRNA in Japanese rice fish embryogenesis is developmentally regulated

While searching for information for *cnr* genes of Japanese rice fish in public databases such as GenBank (www.ncbi.nlm.nih.gov/genbank/) and Ensembl (www.ensembl.org) we found that there are two paralogous *cnr1* genes and one *cnr2* gene (Table 1) in databases and their loci are distributed in three different chromosomes (*cnr1*s are in chromosomes 24 and 22, and *cnr2* is in chromosome 16). The deduced amino acid residues are 469 for cnr1a, 472 for cnr1b and 305 for cnr2. Amino acid identity analysis (Huang and Miller, 1991) showed 64.1% identity between cnr1a and cnr1b, while cnr1a showed 44.9% and cnr1b showed 44.4% amino acid identity with cnr2 receptor. Further analyses indicate that cnr1a and cnr1b showed 70.9% and 60.1% amino acid identity with human CNR1, respectively. However, cnr2 of Japanese rice fish showed only 48.1% amino acid identity with human CNR2 (Table 1). We termed these *cnr1* genes as *cnr1a* (locus on chromosome 24) and *cnr1b* (locus on chromosome 22) with regard to their amino acid identity with human CNR1. We performed conserved synteny analysis between Japanese rice fish and human genome to determine whether *cnr1a* and *cnr1b* genes are duplicates resulting from genome duplication. Our analysis suggests that several genes surrounding *CNR1* genes on human chromosome 6 (Hsp 6) display conserved synteny with regions flanking Japanese rice fish *cnr1a* and *cnr1b* on chromosome 24 (Ola24) and 22 (Ola22), respectively (Fig. 1). For the most part, genes on Japanese rice fish chromosome 24 (Ola24) and chromosome 22 (Ola22) display the same order as those on Hsa6 (*CNR1*) suggesting minimal chromosomal rearrangement in this region. The presence of duplicate genes (*akirin2*, *gabrr1*, *gabrr2*, *ankrd6*) on Ola24 and Ola22 which is also common to Has6 indicates that *cnr1a* and *cnr1b* are the result of gene duplication in Japanese rice fish. Therefore, it is assumed that the *cnr1* duplicates in Japanese rice fish segregate into distinct clades with their human orthologs. Analysis of *cnr2* indicates that it is the single ortholog of human *CNR2* on human chromosome 1 (Hsa1) and also preserves colocalization on Japanese rice fish chromosome 16 (Ola16) in the regions around *cnr2*. Further by PCR based technologies, we have successfully amplified a fraction of all three *cnr* genes using RNA templates prepared from medaka embryos with gene-specific primers (Table 2) (IDT, Coralville, IA). Verification of the nucleotide sequences of the amplified PCR products showed more than 99% identity with the nucleotide sequences reported in GenBank or Ensembl. We therefore considered that these PCR products are the amplified product of *cnr* genes of Japanese rice fish.

To gain further insight into the expression of *cnr* genes during Japanese rice fish development, we used qPCR techniques for quantitative determination of *cnr1a*, *cnr1b and cnr2* gene expression at the message level. Our data indicate that mRNA expression patterns of these *cnr*s are gene-specific and developmentally regulated. The mRNA of *cnr1a* was at the minimal level in the fertilized eggs (Iwamatsu stage 10) and increased gradually with the advancement of morphogenesis and achieved peak level in 6 dpf (enhanced 1892-fold compared to the embryos of 0 dpf) (Fig. 2.1). In the case of *cnr1b*, the mRNA expression pattern is more or less identical to *cnr1a*, however, there are differences. Like *cnr1a*, the embryos at early stages of embryogenesis (0–2 dpf) have low *cnr1b* mRNA copy compared to the embryos of late stages (3–6 dpf). The expression of *cnr1b* mRNA reached maximal level in 4 dpf embryos (41-fold enhancement compared to 0 dpf embryos) and then tended to decrease in 6 dpf embryos (Fig. 2.2). Further significant reduction was observed in hatchlings and *cnr1b* mRNA reached the same level in hatchlings as in the embryos in early stages of development (0–3 dpf) (Fig. 2.2). The *cnr2* mRNA showed maternal expression (highest level in 0 dpf embryos) in Japanese rice fish embryos and followed a different trend than *cnr1a* and *cnr1b* mRNAs. The *cnr2* mRNA decreased to the minimal level in 3 dpf embryos in comparison with 0 dpf embryos and then increased gradually, reaching the highest level in hatchlings (8-fold enhancement compared to 3 dpf embryos) (Fig. 2.3).

WMISB analyses (Fig. 3) of hatchlings (within 24 h of hatching) indicate that signal for *cnr1a* is restricted in the head (brain) region of medaka (Fig. 3.1). Other regions of the body did not show any significant distinction. The signals of the other two *cnr*s are also not very prominent in medaka hatchlings even though *cnr1b* (Fig. 3.2) showed expression in the head (brain) region and *cnr2* (Fig. 3.3) in the pigments distributed in the head region.

3.2. Ethanol is able to alter cnr1a mRNA expression pattern in Japanese rice fish embryogenesis

Fertilized Japanese rice fish eggs were exposed to 300 mM of ethanol either for 48 h (0–2 dpf) (Fig. 4) or for 144 h (0–6 dpf) (Fig. 5) and used for *cnr* mRNA analysis by qPCR. Some of the control and ethanol-treated (300 mM, 0–2 dpf) embryos were further maintained in clean hatching solution (no ethanol) for another 4 days (until 6 dpf) after their removal from ethanol treatment and then used for RNA extraction and analysis.

Table 1
Percentage of amino acid identity between *Oryzias latipes* and *Homo sapiens* CNR homologs.

	Oryzias latipes			*Homo sapiens*	
	cnr1a	cnr1b	cnr2	CNR1	CNR2
Ensemble number	ENSORLT 00000014350	ENSORLT 00000018487	ENSORLT 00000018529	ENST 00000537554	ENST 00000536471
Chromosome loci	24	22	16	6	1
Amino acid residues	469	472	305	472	360
cnr1a (*Oryzias latipes*)	–	64.1%[a]	44.9%[a]	70.9%[a]	47.3[a]
cnr1b (*Oryzias latipes*)	–	–	44.4%[a]	60.1%[a]	42.4%[a]
cnr2 (*Oryzias latipes*)	–	–	–	46.9%[a]	48.1%[a]
CNR1 (*Homo sapiens*)	–	–	–	–	49.6%[a]

[a] Amino acid identity percentages among each couple of homologs (Huang and Miller, 1991).

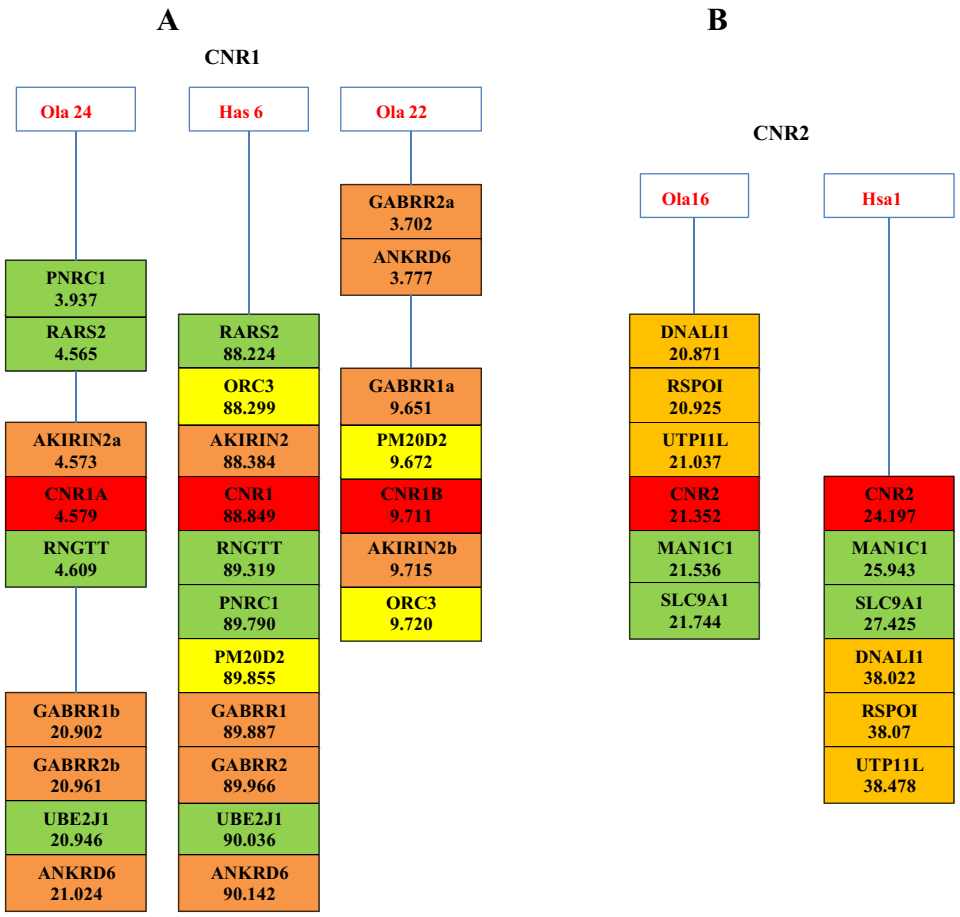

Fig. 1. Genomic analysis of conserved syntenies for Japanese rice fish *cnr* genes. Chromosomal regions surrounding the human (Has) and the Japanese rice fish (Ola) *cnr* genes are represented with genes in boxes and their approximate locations in megabases (Mb). A: *cnr1a* maps to Japanese rice fish chromosome 24 (Ola 24) where as *cnr1b* maps to Ola 22 with several additional duplicates showing co-conserved synteny of these chromosomal segments. B: *cnr2* maps to Ola 16 and is near several genes whose orthologs appear on human chromosome 1 (Hsp 1) near *CNR2*. Gene names and locations indicated as megabases were determined using the Ensembl data base (www.ensembl.gov). *cnr* orthologs are highlighted in red boxes. In figure A, the genes highlighted by green boxes are common to both Has6 and Ola24, those highlighted in yellow are common to Hsa6 and Ola22, and those in orange are common to Hsa6 as well as Ola24 and Ola22. In B the genes highlighted by green boxes have followed the same orientation in both Ola16 and Hsa1 with respect to *CNR2*, however, the genes highlighted in orange boxes showed differences in orientation (in Ola16 upstream of *cnr2* and in Hsa1 downstream to *CNR2*).

Microscopic examination of the embryos after ethanol removal in 2 dpf showed that ethanol (300 mM) treatment affects the cardiovascular development (delay in vessel circulation and development of thrombi). Maintenance of these embryos for another four days (6 dpf) in hatching solution showed that more than 80% of the embryos were able to initiate vessel circulation (flow of blood inside the blood vessel). Therefore, we have excluded those ethanol-treated embryos (20% or less) from the experiments that were unable to initiate vessel circulation in 6 dpf. In contrast, the embryos when exposed to ethanol (300 mM) continuously for six days (0–6 dpf) the deformities appear to be more and none of the embryos were able to initiate vessel circulation. Moreover, the pigmentation of the eyes was affected and thrombi were found throughout the body, especially in the blood island (data not shown). However, the survivability of the embryos was more than 80%. We used all the surviving embryos for RNA extraction despite the lack of vessel circulation.

It was observed that embryos (Iwamatsu stage 10) exposed to ethanol (300 mM) for 2 days (2 dpf) were unable to show any significant effect on mRNA content of any of these *cnr* genes (*cnr1a*, *1b* and *2*) when compared with the corresponding control embryos (maintained in hatching solution) (Fig. 4). Removal of ethanol from the medium and maintenance of the embryos in hatching solution until 6 dpf (another four days) showed that the embryos were able to enhance *cnr1a* mRNA expression in both control and ethanol-treated embryos (enhancement in controls is 333-fold compared to 2 dpf embryos whereas in ethanol treated embryos it is only 37-fold). However, the enhancement in 6 dpf was significantly less in ethanol-treated embryos than control embryos (Fig. 4.1). Under identical conditions (exposed to 300 mM of ethanol 0–2 dpf and then maintained in hatching solution until 6 dpf without ethanol), the other two mRNAs (*cnr1b* and *cnr2*) also showed enhanced levels in 6 dpf embryos when compared with the 2 dpf embryos. However, comparison of the *cnr1b* or *cnr2* mRNA

Table 2
List of primers used for amplification of *cnr* genes in Japanese rice fish.

Name of the gene	Sense (5′–3′)	Antisense (5′–3′)	Product (bp)	Ensembl number
cnr1a	cccaatcatctacgccttgag	gcttctgcagaagtttcggttg	224	ENSORLT00000014350
cnr1b	gctcccagaggagcgtgatcg	cgcaggtccttgctcctcatg	279	ENSORLT00000018487
cnr2	ccgccatgaatcctccatggc	cagcgcagtccatagaggag	257	ENSORLT00000018529

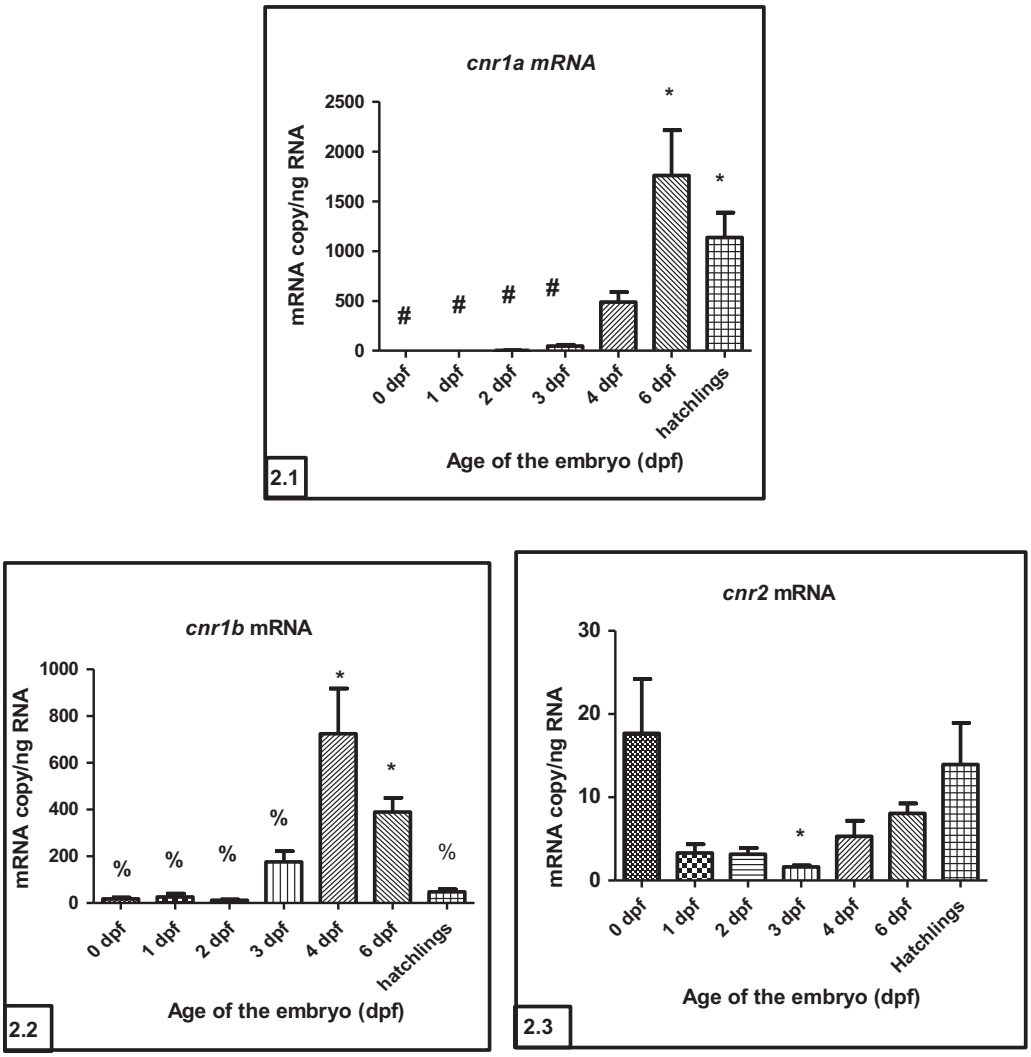

Fig. 2. Developmental regulation of the *cnr* genes of Japanese rice fish embryos during development analyzed by qPCR. 2.1 = *cnr1a*, 2.2 = *cnr1b*, 2.3 = *cnr2*. The embryos were collected within ~2 hpf and maintained under laboratory conditions (26 ± 1 °C; 16L:8D light cycle) in hatching solution. Each bar is the mean ± SEM of four observations. The data were analyzed by one way ANOVA followed by post-hoc Tukey's multiple comparison test: $p < 0.05$ was considered significant. Asterisks (*), pound (#), or percent (%) symbols indicate that the data are significantly different from 0 dpf (*), 4 dpf (%) or 6 dpf (#) embryos. dpf = days post fertilization.

content between control and ethanol-treated embryos in 6 dpf did not establish any significant difference (Fig. 4.2 and 4.3).

To evaluate whether the presence of ethanol in the medium is necessary to produce any significant effects on *cnr* gene expression, we exposed the embryos continuously to 300 mM of ethanol for 6 days (0–6 dpf) of development (Fig. 5). The mRNA analysis on 6 dpf showed that only *cnr1a* mRNA decreased significantly in comparison with the controls (Fig. 5.1), while *cnr1b* and *cnr2* mRNAs maintained the same level as in controls (Fig. 5.2 and 5.3).

3.3. Reduction of cnr1a mRNA by ethanol is concentration dependent

We further evaluated a concentration dependent effect of ethanol on *cnr* mRNA expression in Japanese rice fish embryos on 6 dpf. The embryos were exposed to 100–500 mM ethanol 0–2 dpf and then cultured in clean hatching solution without ethanol for another 4 days (6 dpf) and used for RNA extraction and analysis. Parallel control embryos were maintained in identical conditions in hatching solution without ethanol. It was observed that embryos exposed to 300 mM and higher concentrations (300–500 mM) of ethanol were able to significantly reduce *cnr1a* mRNA content in 6 dpf when compared with control embryos. However, embryos treated with lower doses of ethanol (100–200 mM) maintained the same level of *cnr1a* mRNA as in controls in 6 dpf (Fig. 6.1). Further analysis indicates that the level of *cnr1a* mRNA reduction by higher concentration of ethanol (300–500 mM) is more or less equal (unable to establish any significant difference between these three concentrations). Other two mRNAs (*cnr1b* and *cnr2*) under identical conditions of ethanol treatment (100–500 mM) also maintained the same levels of mRNAs as in control embryos 6 dpf (Fig. 6.2 and 6.3).

3.4. Effects of rimonabant and AM-251 on FASD phenotypic features of medaka

Medaka embryos (Iwamatsu stage 10) were exposed to CB1 receptor antagonists, rimonabant (10–20 μM) or AM-251 (0–2–1 μM), for 0–2 dpf and evaluate the phenotypes during embryogenesis (heart morphology, vessel circulation and thrombus formation) or in hatchlings (within 24 h of hatching). In another experiment 0 dpf embryos (Iwamatsu stage 10) were exposed to AM 251 (1 μM) continuously from 0–6 dpf and then transferred to the hatching solution. It was observed that neither rimonabant nor AM-251 in 0–2 dpf exposure was able to alter heart morphology (all looped) and vessel circulation or produce any thrombus during embryogenesis. The hatching efficiency

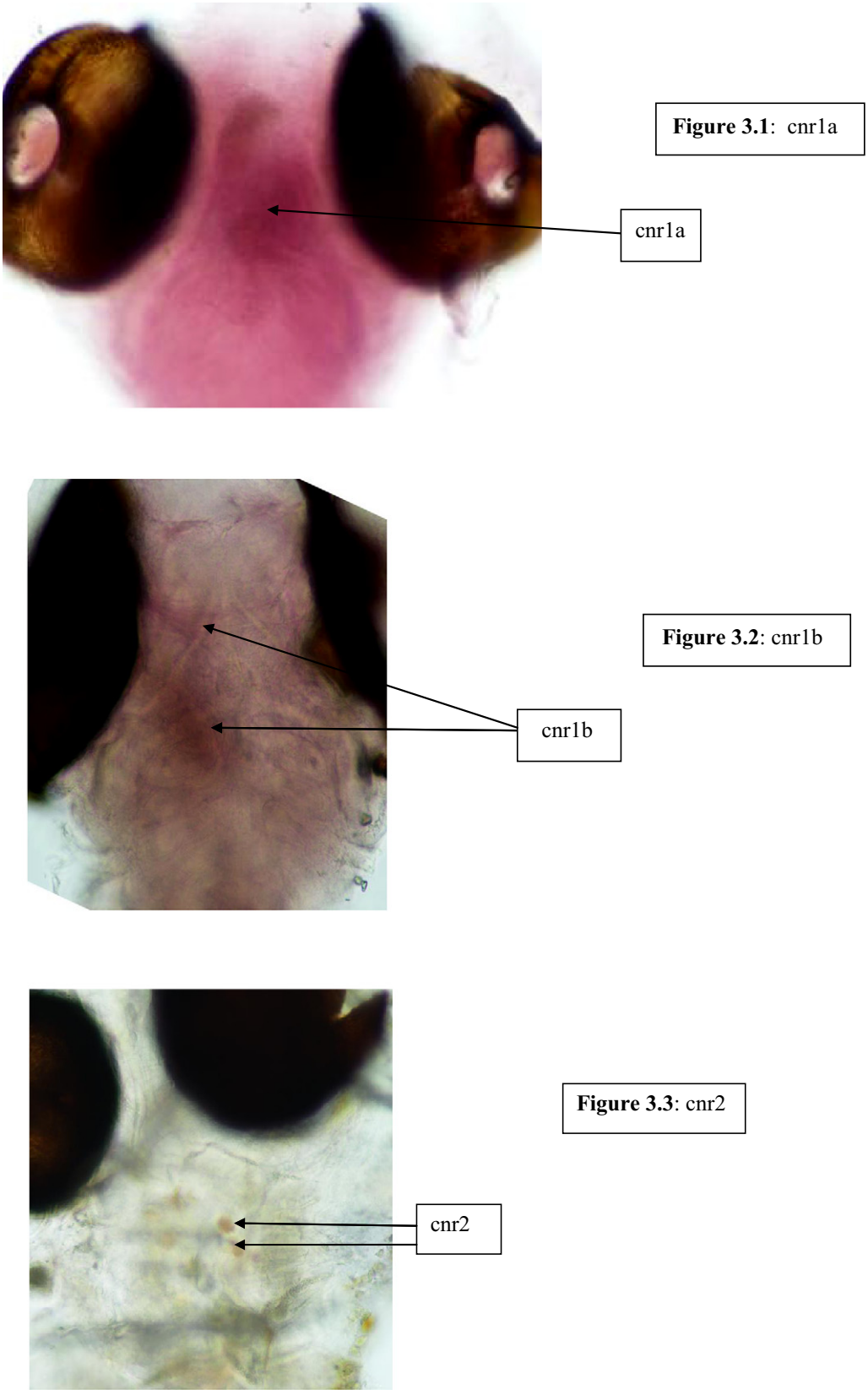

Fig. 3. Whole mount in-situ hybridization (WMISH) analysis of *cnr1a*, *cnr1b*, and *cnr2* mRNAs of Japanese rice fish hatchlings. The hatchlings were collected within 24 h of hatching and WMISH analyses were done after Inohaya et al. (1995). The expression of all three *cnr* mRNAs was restricted in the head region of the hatchlings. 3.1 = *cnr1a*, 3.2 = *cnr1b* and 3.3 = *cnr2*.

and the craniofacial deformities remained unaltered as observed in control embryos (Table 3). Continuous exposure of the embryos to AM 251 was also unable to induce any cardiovascular and craniofacial deformities, however, only the hatching efficiency was reduced in embryos exposed to AM 251 when compared with the corresponding controls (Fig. 7).

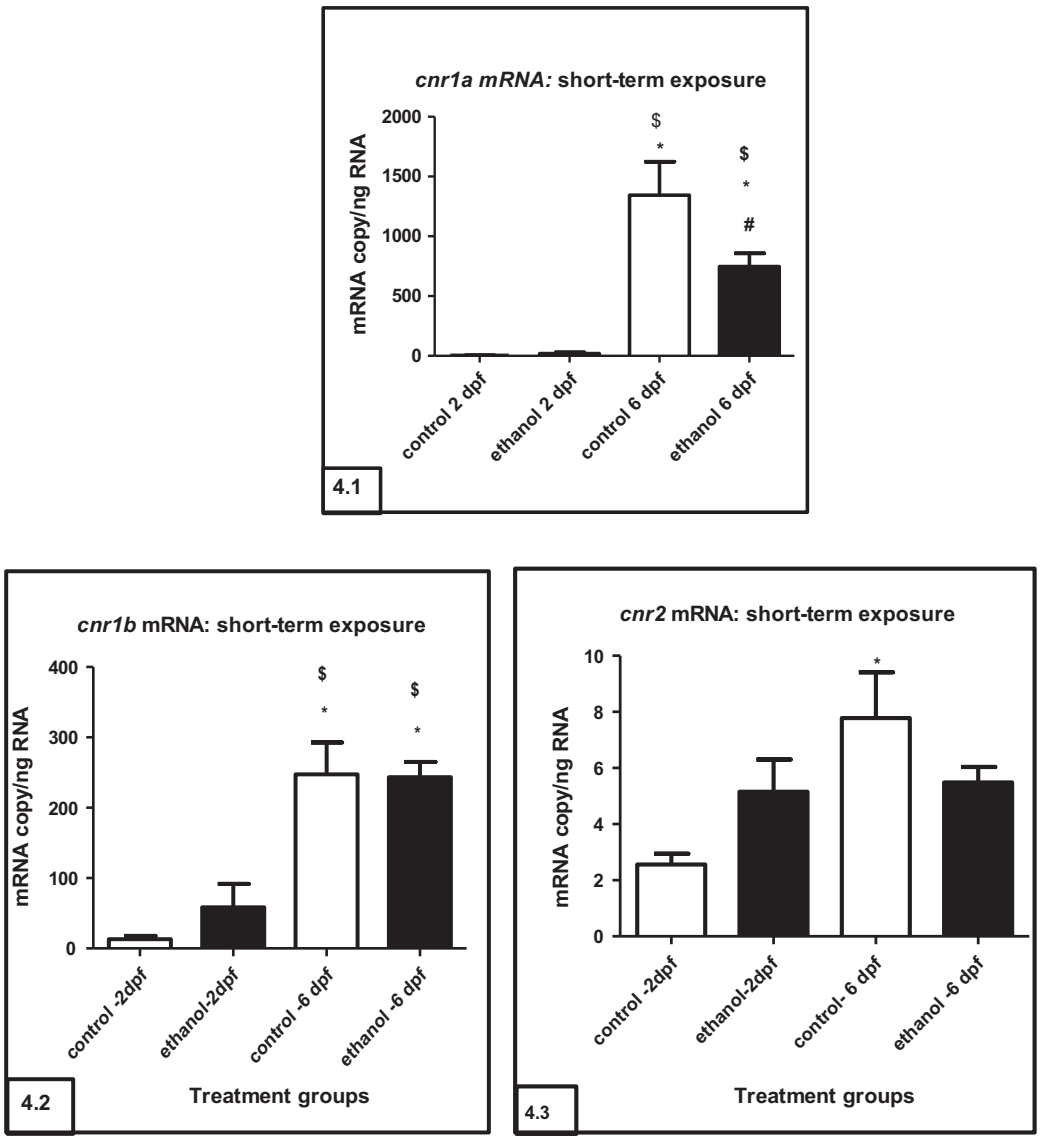

Fig. 4. Effect of developmental ethanol exposure (short period) on the *cnr* mRNA contents of Japanese rice fish embryos. 4.1 = *cnr1a*, 4.2 = *cnr1b*, 4.3 = *cnr2*. Fertilized eggs (Iwamatsu stage 10) were exposed to 300 mM ethanol 0–2 dpf and analyzed either on 2 dpf after removal of ethanol or maintained in clean hatching solution 6 dpf and analyzed. Each bar is the mean ± SEM of 4–6 observations. The data were analyzed by one way ANOVA followed by post-hoc Tukey's multiple comparison test. $p < 0.05$ is considered significant. Empty bar = control; filled bar = ethanol. Asterisks (*) indicate that the data are significantly different from the 2 dpf control, a dollar sign ($) indicates difference from the 2 dpf ethanol treatment, and the pound (#) symbol indicates that the data are significantly different from 6 dpf control.

4. Discussion

The present study was focused on endocannabinoid receptors of Japanese rice fish as a probable target of ethanol for inducing FASD phenotypes. To get insight into the system, we followed four strategies. We first collected information about *cnr* genes of Japanese rice fish from the public data bases for comparison with human CNR and then analyzed their expression pattern during development and evaluated any modulation caused by developmental ethanol exposure (both short and long-time duration). Finally, we have exposed the embryos to CB1 receptor antagonists (rimonabant or AM 251) and evaluated any development of FASD phenotypic features we characterized previously (Haron et al., 2012). Analyses of amino acid residue identity (Table 1) and synteny (Fig. 1) with human CNR sequence indicate that the Japanese rice fish genome has two paralogous *cnr1* and one *cnr2*. In comparison with human CNR1 of the amino acid residues, Japanese rice fish cnr1 showed that cnr1 locus in Ola24 (*O. latipes* chromosome 24) has more amino acid residue identity than the locus in Ola22 (*O. latipes* chromosome 22) (Table 1). Further, the synteny analyses of *cnr1* on Ola24 and *cnr1* on Ola22 with human *CNR1* on Hsp6 (*Homo sapiens* chromosome 6) indicate that Japanese rice fish like *Fugu rubripes* has two *cnr1* paralogs in the genome (Yamaguchi et al., 1996). The presence of duplicate genes in the fish genome is very common because during evolution, the fish line underwent genome duplication after separation from the tetrapod lines (Amores et al., 1998). Considering the amino acid residue identity of cnr1 in Japanese rice fish with human CNR1, we termed the cnr1 locus on Ola24 as *cnr1a* and the locus on Ola 22 as *cnr1b*. Once we verified the structural identity, our next aim was to verify how these two cnr1 paralogs are functionally similar.

Our data further indicated that all three *cnr* expressions were developmentally regulated while *cnr*2 showed maternal inheritance. The expression of *cnr1a* and *cnr1b* was at the minimal level in the embryos 0–3 dpf and attained peak level later in prehatching stages (1892-fold increase in *cnr1a* in 6 dpf embryos and 41-fold increase in *cnr1b* in 4 dpf embryos compared to 0 dpf). However, in hatchlings, *cnr1a* maintained the same status as in 6 dpf, but *cnr1b* was significantly reduced (Fig. 2.1 and 2.2). Due to maternal expression, *cnr2* showed the highest mRNA copy in 0 dpf, reduced until 3 dpf and then increased gradually and

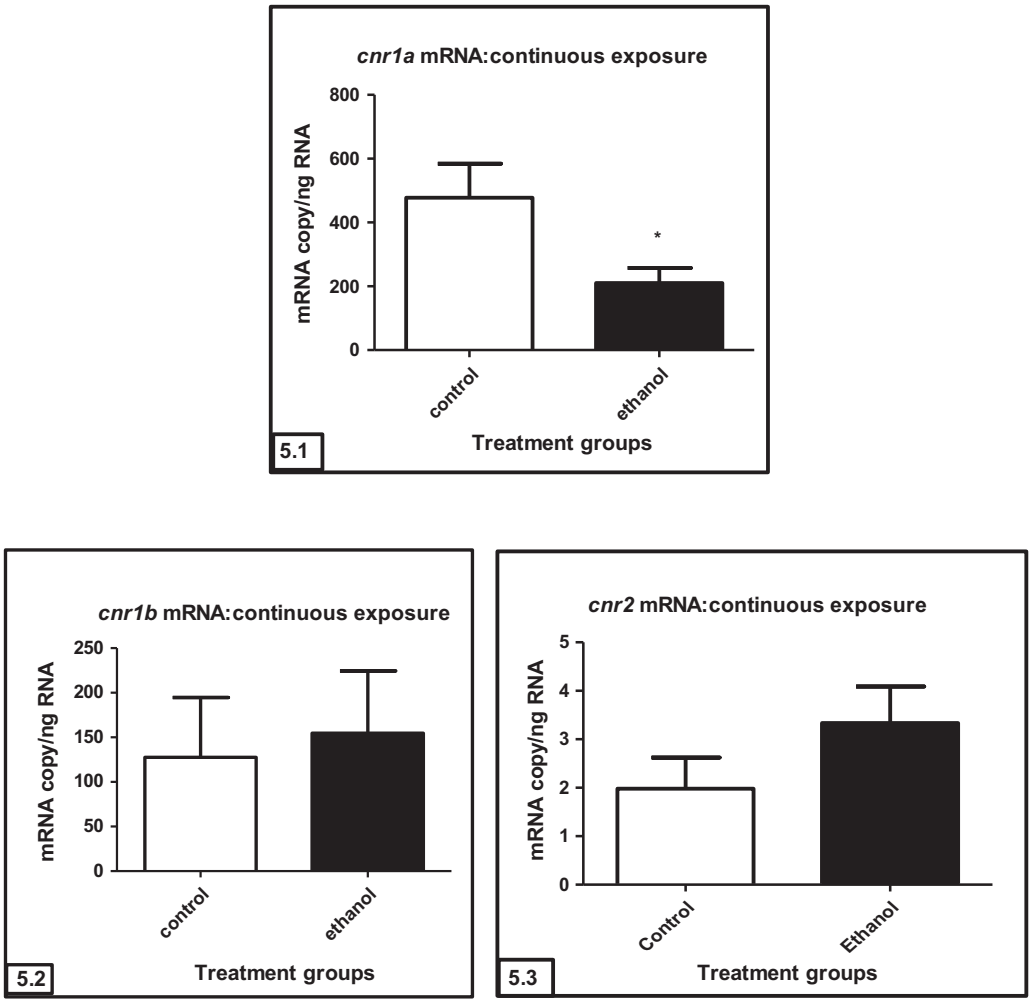

Fig. 5. Effect of continuous ethanol (continuous six days) exposure on the *cnr* mRNA content of Japanese rice fish embryos. 4.1 = *cnr1a*, 4.2 = *cnr1b*, 4.3 = *cnr2*. Fertilized eggs (Iwamatsu stage 10) were exposed to 300 mM ethanol 0–6 dpf and analyzed. Each bar is the mean ± SEM of 6 observations. The mean data were determined by GraphPad prism version 5 and the level of significance was determined by Student's unpaired 't' test; $p < 0.05$ is considered significant. Empty bar = control; filled bar = ethanol. Asterisks (*) indicate that the data were significantly different from the corresponding controls.

continued increasing even after the embryos were hatched (Fig. 2.3). WMISH analysis indicated that in hatchlings all *cnr* mRNAs were expressed in the head (brain) region and *cnr1a* expression was comparatively more prominent than the others (Fig. 3). Our analysis further indicated that ontogenesis of *cnr1* and *cnr2* in Japanese rice fish is more or less similar to that of humans or other vertebrates where *Cnr1* expression was detected very early during gestation (analysis was done either on mRNA or protein). In zebrafish, qPCR analysis showed that *cnr1* was detected as early as in the three somite stages (12 hpf) and then increased with the advancement of development except in 25 somite stages (24 hpf) (Migliarini and Carnevali, 2009). Moreover, although *cnr1* was expressed in mature oocytes of zebrafish, it was not inherited by the offspring during fertilization. In situ hybridization analysis in zebrafish embryos also revealed further that appearance of *cnr1* first occurred in the preoptic area at 24 hpf (Lam et al., 2006) and continued to express in the embryos at 48 and 72 hpf. In humans and rodents the expression of *Cnr1* occurred very early during brain development and the expression persisted throughout development (Buckley et al., 1997; Zurolo et al., 2010). CNR2 was initially identified as a peripherally restricted receptor predominantly expressed in cells of the rodent and human immune system (Munro et al., 1993; Lynn and Herkenham, 1994; Galiegue et al., 1995; Schatz et al., 1997), and was not expressed in the human brain during gestational weeks 9–36 (Zurolo et al., 2010). In rats, in situ hybridization analysis showed that *Cnr2* expression exclusively occurred in the liver of the embryos as early as gestation day 13 (Buckley et al., 1997). Although the WMISH analysis of Japanese rice fish hatchlings by us indicated expression of all three *cnr* genes in the brain region (Fig. 3), from our qPCR (Fig. 2) data we have reason to believe that in Japanese rice fish endocannabinoid receptors emerge early during brain development and followed almost identical ontogenic patterns as in humans and other vertebrates. Moreover, ontogenesis of *cnr1a* and *cnr1b* followed the same patterns which were different from the *cnr2* pattern.

Further, we aim to study the response of all three *cnr*s to developmental ethanol exposure that may lead to analogous FASD phenotypes in Japanese rice fish. As ethanol perturbs embryonic development of Japanese rice fish, we expect that ethanol also disrupts the expression of *cnr* genes and leads to induction of FASD phenotypes. A strong interaction has been elucidated between ethanol and the components of the EC system (Pava and Woodward, 2012) and also in the development of alcohol abuse and dependence (Erdozain and Callado, 2011). Moreover, a recent report shows that mice exposed to ethanol during neurodevelopment have reduced expression of *Cnr1* and a concurrent increase in regulatory microRNA (*miR-26b*) in the adult brain that may ultimately be able to explain the molecular mechanisms of FASD (Stringer et al., 2013). In a study on transgenic zebrafish, chronic ethanol treatment (0.5–1.5%) during embryonic development (6 hpf to 36 hpf) reduces CB1R (*cnr1*) expression and motor neurogenesis in a concentration-dependent manner and the effects are attenuated by CP55940 (CB1R agonist) or URB597 (FAAH-inhibitor) cotreatment

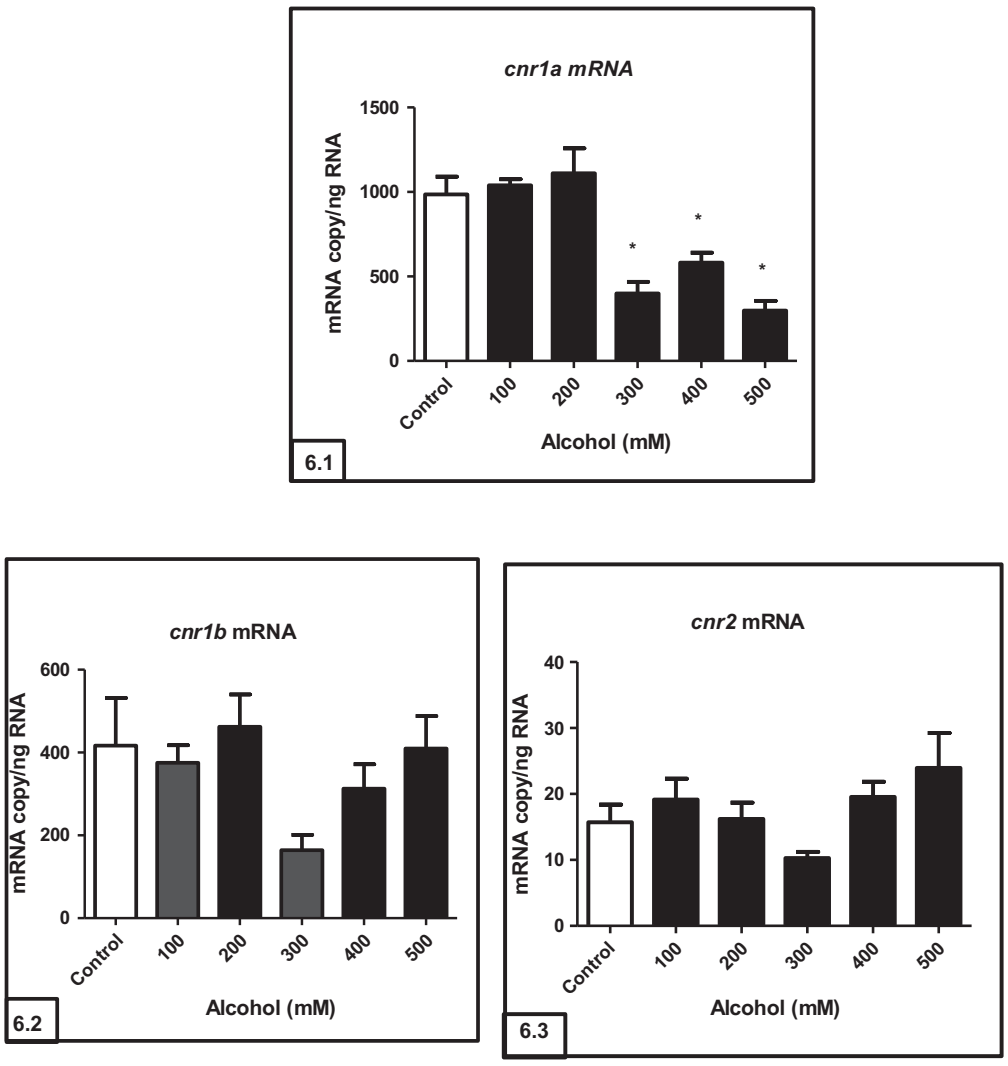

Fig. 6. Effects of different concentrations of ethanol (100–500 mM) on the expression of cnr mRNAs of Japanese rice fish embryos on 6 dpf. Fertilized rice fish eggs (Iwamatsu stages 9–10) were exposed to different concentrations of ethanol (100–500 mM) for 48 h and then maintained in clean hatching solution until 6 dpf. Parallel controls were maintained in hatching solution with no ethanol. RNA was extracted, reverse transcribed and utilized for *cnr* mRNA analysis by qPCR. Each bar is the mean ± SEM of 4–6 observations. The data were analyzed by one way ANOVA followed by post-hoc Tukey's multiple comparison test. $p < 0.05$ is considered significant. Empty bar = control; filled bar = ethanol. Asterisks (*) indicate that the data are significantly different from control. 6.1 = *cnr1a*, 6.2 = *cnr1b* and 6.3 = *cnr2*. It was observed that only *cnr1a* expression reduced significantly in the embryos treated with 300–500 mM of ethanol.

(Mukhopadhyay et al., 2011). To confirm our hypothesis, initially we have exposed the embryos (Iwamatsu stage 10) to 300 mM of ethanol for 48 h followed by analyses of *cnr* mRNAs by qPCR immediately after ethanol removal (2 dpf) or maintained another 4 days in hatching solution (6 dpf) and used for *cnr* mRNA analysis. We have used this ethanol concentration (300 mM) for several reasons. First of all, the morphological abnormalities of Japanese rice fish we considered for evaluation of FASD phenotypes are always reproducible at this concentration (Hu et al., 2009; Haron et al., 2012). Moreover, the embryonic ethanol concentration in Japanese rice fish is ~34% of the waterborne ethanol if the ethanol concentration is 300 mM in the medium (Haron et al., 2013). Therefore, the calculated embryonic ethanol concentration would be 102 mM which is in reasonable agreement with mammalian models (required ethanol concentration to induce FASD phenotypes is

Table 3
Evaluation of FASD phenotypes in medaka embryos and hatchlings under different treatment conditions.

	Treatment conditions	Vessel circulation	Thrombus	Heart morpho logy	Hatching efficiency	NCL	TC	PC
Control	Clean hatching solution	Initiates 50 hpf	Not observed	Looped	75% or more embryos hatched by 10 dpf	Un-altered	Normal	Normal
Ethanol	300 mM (0–2 dpf)	Delayed	Observed	25–50% of the embryos have tube heart	Delayed	Reduced	Deformed	Deformed
Rimo-nabant	10–20 μM (0–2 dpf)	Similar to controls	Not observed	Looped	Same as control	Un-altered	Un-altered	Un-altered
AM 251	0.2–1 μM (0–2 dpf)	Similar to controls	Not observed	Looped	Same as control	Un-altered	Un-altered	Un-altered
AM 251	1 μM (0–6 dpf)	Similar to controls	Not observed	Looped	Delayed	Un-altered	Un-altered	Un-altered

Fertilized medaka eggs (Iwamatsu stage 10) were exposed to ethanol, rimonabant and AM 251 for various time points and the embryos were examined for heart morphology, thrombus formation and vessel circulation. When the embryos were hatched, they were preserved in 4% paraformaldehyde within 24 h of hatching and examined for neurocranial deformities (Hu et al., 2009) after Alcian blue staining (Haron et al., 2013). NCL = Linear length of neurocranium, TC = Trabecular cartilage, PC = Polar cartilage.

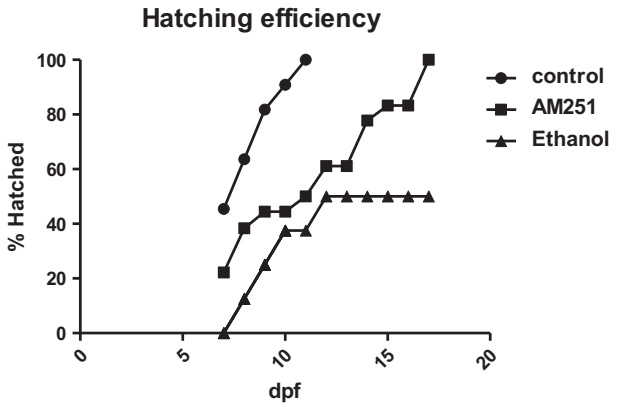

Fig. 7. Representative data showing the hatching efficiency of the embryos exposed to AM 251 (1 μM, 0–6 dpf) or ethanol (300 mM, 0–2 dpf). The controls were maintained in hatching solution containing 0.01% DMSO. Hatching was delayed in embryos treated with AM 251 or ethanol. dpf = days post fertilization.

88 mM, Liu et al., 2009). Furthermore, the LD_{50} of ethanol we have determined previously for Japanese rice fish is 568 mM (Wang et al., 2006) which is almost twice the ethanol concentration (300 mM) we used for FASD induction in Japanese rice fish.

Our data indicated that ethanol (300 mM) while inducing FASD phenotypes in Japanese rice fish was able to reduce only *cnr1a* mRNA content in 6 dpf embryos irrespective of the duration of ethanol treatment (either 2 dpf or 6 dpf) in the medium (Figs. 4.1 and 5.1). A concentration-dependent analysis of the expression of *cnr* mRNAs on 6 dpf embryos after exposing them to five different concentrations (100–500 mM) of ethanol for the first 48 h of development (0–2 dpf) also indicated that ethanol concentration of 300 mM and above was able to reduce only *cnr1a* mRNA expression (Fig. 6.1). Despite significant structural identity of *cnr1b* gene with *cnr1a*, both *cnr1b* and *cnr2* RNAs remained unaffected and maintained the normal developmental rhythm of expression even in the continuous presence of ethanol in the medium or with the increase of ethanol concentrations to 500 mM in short exposure (Figs. 4.2, 4.3, 5.2, 5.3, 6.2 and 6.3). However, in continuous ethanol exposure experiments the embryos were maintained in tightly capped 2 ml tubes with daily replacement of the media. In these conditions, all *cnr* mRNA levels in both control and experimental embryos on 6 dpf were found to be reduced when compared with those of the 6 dpf embryos maintained in glass bowls and allowed normal development (Fig. 2). Although it is unexpected, we predict that continuous maintenance of the embryos in the tightly capped tubes may induce stress that may delay the normal developmental processes which were reflected in the mRNA contents of all three *cnr* mRNAs. Although there are variations in control values, the nature of changes we observed remained the same (all *cnr* mRNAs were enhanced in 6 dpf embryos when compared with the 2 dpf embryos or significant reduction of *cnr1a* mRNA in 6 dpf embryos exposed to 300–500 mM of ethanol 0–2 dpf when compared with control embryos on 6 dpf). Therefore, the reduced level of *cnr1* mRNA in Japanese rice fish by developmental ethanol exposure is probably an important indicator of the induction of FASD phenotypes. Other *cnr1* paralog (*cnr1b*), although present in the Japanese rice fish genome, did not show any significant response to embryonic ethanol exposure. Therefore, we may infer that *cnr1a* not the *cnr1b* of Japanese rice fish genome is the ancestral form of mammalian *CNR1* that responds to ethanol.

Previously we have observed that ethanol was able to disrupt the cardiovasculature of Japanese rice fish embryogenesis which we hypothesized as one of the potential causes of induction of FASD phenotypes (Hu et al., 2008). Moreover, modulation of Cnr1 expression by a specific agonist or antagonist is also able to alter blood pressure and heart rate and can induce atherosclerosis (Gadde and Allison, 2006; Steffens and Mach, 2006). Therefore, it is possible that abnormal cardiovasculature in Japanese rice fish embryos as induced by ethanol is the result of down regulation of *cnr1a* that ultimately leads to FASD phenotypes. To verify this concept, we exposed the embryos to CB1 receptor antagonists (rimonabant and AM 251) and examined embryos and hatchlings for FASD phenotypes. We predict that these CB1 receptor antagonists can block cnr1a receptor activities and are likely to induce FASD phenotypes. In zebrafish AM 251 was able to reduce the hatching rate of the embryos and motility behavior in hatchlings (Migliarini and Carnevali, 2009; Akhtar et al., 2013). Our studies showed that short-term exposure (0–2 dpf) of the embryos either to rimonabant (10–20 μM) or to AM 251 (0.2–1 μM) was unable to induce any developmental disorders in Japanese rice fish embryos (Table 3). However, continuous exposure (0–6 dpf) of the embryos to AM 251 (1 μM) was able to induce hatching delay (Table 3, Fig. 7). Other FASD phenotypic features (tube heart, delay in vessel circulation, thrombus formation, microcephaly, trabecular and polar cartilage deformities) as identified by us (Hu et al., 2009) are absent both in embryos and hatchlings (Table 3). Although in this study we did not include any behavioral parameters which are considered indicators of neurological disorders, we believe that blocking of cnr1a by antagonists may induce neuronal disorders that lead to neurobehavioral disorders observed in FASD rather than inducing any observable morphological defects. This study therefore further indicates that the morphological disorders induced by ethanol in Japanese rice fish embryogenesis as observed by us in our FASD studies are probably not regulated by cnr1-mediated mechanisms. However, the role of cnr1 with regard to neurological disorders as observed in FASD needs further evaluation in this model possibly by knocking down or overexpressing *cnr1a* gene.

Taken together, these data on Japanese rice fish open further opportunity to identify *cnr1a* gene as a probable target of ethanol which may have some role in the development of FASD. The other paralogs *cnr1b* and *cnr2* are simply expressed in the embryos and play no role in ethanol-mediated toxicity.

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